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Carbachol and bradykinin elevate cyclic AMP and rapidly deplete ATP in cultured rat sympathetic neurons

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The agonists carbachol (CCh) and bradykinin (BK) and 54 mM KCl (high K^+) were among the most potent stimulants of cyclic AMP (cAMP) production in cultured rat sympathetic neurons, measured with the use of a high-fidelity assay developed for small samples. The rise in cAMP evoked by CCh (through muscarinic receptors), BK, and high K^+ was inhibited in Ca^{2+} -depleted medium (1.3 mM Ca^{2+} and 2 mM BAPTA or EGTA), which also prevented the sustained rise in $[Ca^{2+}]_i$ evoked by each of these stimuli, showing that elevation of cAMP requires extracellular Ca^{2+} and, possibly, Ca^{2+} influx. Preliminary results obtained with the novel calmodulin inhibitor CGS 9343B, which blocked the elevation of cAMP, and with the cyclooxygenase inhibitor indomethacin, which partially blocked the actions of the agonists but not those of high K^+ , suggest that calmodulin and arachidonate metabolites may be two components of the signaling pathway. In addition to their effects on cAMP metabolism, CCh, muscarine, and BK, but not nicotine, caused a 30–40% decrease in ATP levels. This effect was much greater than that evoked by high K^+ and was largely inhibited by CGS 9343B but slightly enhanced in the Ca^{2+} -depleted medium, showing that agonists are still active in the absence of $[Ca^{2+}]_o$. Thus, agonists that activate phosphoinositide metabolism can also increase cAMP production and substantially deplete cells of ATP. These novel actions may have to be taken into account when the mechanisms by which such agonists regulate cell function are being considered.

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Introduction

Cyclic AMP (cAMP) recently has been shown to enhance the survival of cultured superior cervical ganglion (SCG)¹ neurons in the absence of nerve growth factor (Rydel and Greene, 1988)—a neurotrophic agent required by these cells for survival (Levi-Montalcini, 1987)—suggesting that cAMP may be a physiologically relevant modulator of neuronal survival. It is therefore important to establish the stimulants that promote elevation of neuronal cAMP. Little is known about which stimulants cause a rise in cAMP in SCG neurons *in vivo*. In the isolated rat SCG *in vitro*, the most potent stimulants of cAMP production were electrical stimulation (Volle and Patterson, 1982), β -adrenergic agonists (Cramer *et al.*, 1973; Brown *et al.*, 1979; Bone *et al.*, 1984), histamine (Lindl and Cramer, 1974), vasoactive intestinal polypeptide (VIP) (Volle and Patterson, 1982) and carbachol (CCh) (Briggs *et al.*, 1982); but the identity of the cells in which the changes in cAMP levels occurred is not known, nor is it clear whether these agents stimulated SCG neurons directly or indirectly through mediators released by preganglionic nerve terminals or other cells in the ganglion. In cultured SCG neurons Walicke and Patterson (1981a,b) observed elevated cAMP levels after several days of exposure to prostaglandin E_1 (PGE1) or 20 mM KCl, but agonists such as CCh or bradykinin (BK) were reported to be without effect, perhaps because of the long periods of exposure. We screened several

¹ Abbreviations: ACh, acetylcholine; BAPTA, 1,2 bis(2-aminophenoxy)ethane *N,N,N,N'* tetraacetic acid; BK, bradykinin; CaM, calmodulin; cAMP, cyclic AMP; CCh, carbachol; CGS-9343B, 1,3 dihydro-1-[1-[(4-methyl-4H,6H pyrrolo[1,2-a][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2H-benzimidazol-2-one (1:1) maleate; DMSO, dimethyl sulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; high K^+ , 54 mM KCl; IBMX, 3-isobutyl 1-methylxanthine; mAChR, muscarinic ACh receptor; NA, noradrenaline; nAChR, nicotinic ACh receptor; PGE1, prostaglandin E_1 ; PKC, protein kinase C; SCG, superior cervical ganglion; Tris, tris(hydroxymethyl)aminomethane; VIP, vasoactive intestinal polypeptide.

different agents that are likely to be released within the SCG for their ability to raise cAMP in cultured rat SCG neurons devoid of nonneural cells and found the acetylcholine (ACh) analogue CCh and BK to be the most potent among several agents that elevated cAMP (Suidan, 1989). We chose to further investigate their mechanisms of action, as they were previously shown to be without effect and are not known to activate adenylate cyclase through the stimulatory GTP-binding protein Gs (Peralta *et al.*, 1988).

In the present study, cAMP production was followed by metabolic labeling of the neurons with [^3H]adenine (Shimizu and Daly, 1970) by a simple method for nucleotide separation that enables simultaneous determination of cAMP and other adenine-based metabolites. Using this method, we show that CCh and BK, in addition to elevating cAMP and promoting Ca^{2+} influx, also cause a substantial depletion of ATP.

Results

CCh and BK induce cAMP production and ATP breakdown

The effects of CCh (100 μM), BK (10 nM), and 54 mM KCl (high K^+) on cAMP production and AMP/IMP levels after 3 min of stimulation of SCG explants are shown in Figure 1. It should be noted that the increase in AMP/IMP shown was because of an equivalent decrease in ATP, which comprised $\sim 90\%$ of all nucleotides labeled under resting conditions (Tolkovsky and Suidan, 1987). It can be seen that all three stimulants raised cAMP production (Figure 1A) and also caused a substantial increase in AMP/IMP (Figure 1B), with high K^+ causing the largest increase in cAMP and yet the smallest increase in AMP/IMP. The increases in cAMP and AMP/IMP were similarly dependent on the concentration of CCh (Figure 1C), suggesting that the two responses were mediated by stimulation of the same receptor. An effective concentration (EC_{50}) of $8 \pm 2 \mu\text{M}$ for CCh was derived by fitting the data to an equation for a single, saturable binding site. These results were obtained from single-cell cultures, virtually devoid of nonneural cells, showing that the responses to CCh were occurring in the neurons.

The large increase in AMP/IMP caused by the agonists was surprising. A comparison of the relative increases in cAMP and AMP/IMP induced by the three stimulants (Table 1) showed that the changes in AMP/IMP relative to cAMP were highly variable, ranging from 2.8 for BK to 0.7 for high K^+ , suggesting that the rise in AMP/

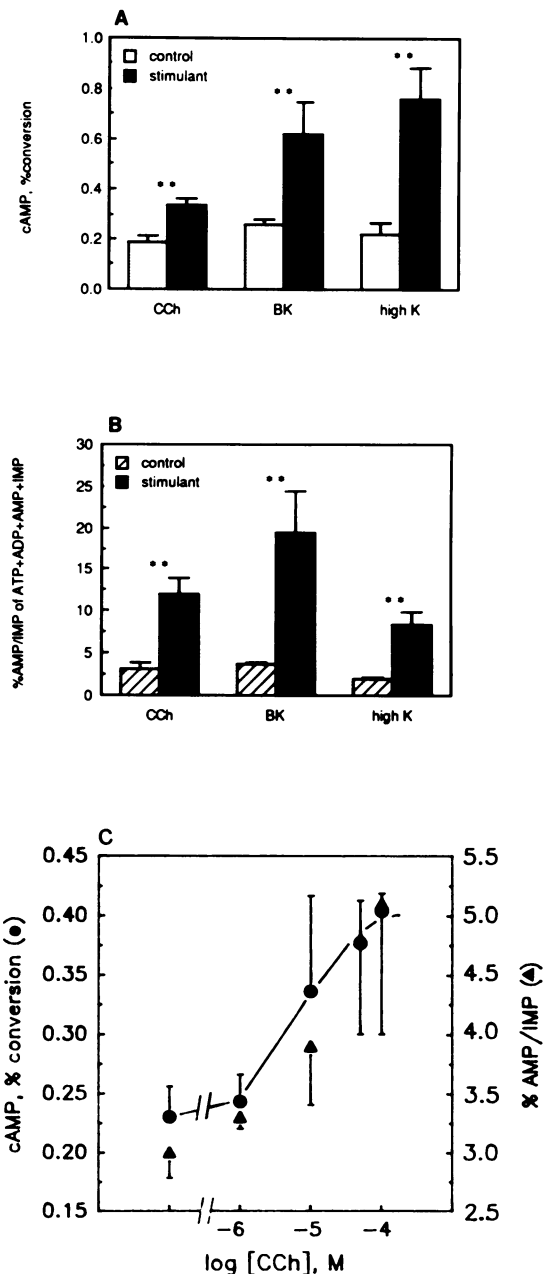


Figure 1. cAMP and AMP/IMP elevation in response to CCh, BK, and high K^+ . Explants were incubated with 1.9 μM [^3H]adenine for 14 h, washed, and incubated for 3 min at 37°C in medium containing 1 mM IBMX; 0.4% DMSO; and either CCh (100 μM), BK (10 nM), or high K^+ (54 mM). Adenine nucleotides and cAMP were separated by thin-layer chromatography, extracted, and analyzed. (A) Percent conversion of cAMP was calculated by dividing the radioactivity incorporated into cAMP by the radioactivity in the total nucleotide pool (ATP, ADP, AMP, and IMP). (B) Percent AMP/IMP was similarly calculated by dividing the radioactivity incorporated into AMP/IMP by the radioactivity in the total nucleotide pool (ATP, ADP, AMP, and IMP). The results are presented as means of triplicates \pm SD, ** $p < 0.005$. (C) A 3-wk culture of single neurons, depleted of nonneural cells, was similarly treated with different concentrations of CCh.

Table 1. Potential of cAMP elevating agents to cause ATP depletion

Agonist	Stimulation (fold)		(AMP/IMP):cAMP ratio
	cAMP	AMP/IMP	
Carbachol (100 μ M)	2.2 \pm 0.4 (6)	4.1 \pm 1.2 (6)	1.9
Bradykinin (10 nM)	3.2 \pm 1.4 (4)	9.0 \pm 2.7 (4)	2.8
High K ⁺ (54 mM)	5.8 \pm 1.8 (3)	3.9 \pm 0.5 (4)	0.7
Isoprenaline (10 μ M)	3.2 \pm 1.1 (2)	1.2 \pm 0.5 (2)	0.4
VIP (10 nM)	2.1 \pm 1.2 (2)	1.3 \pm 0.7 (2)	0.3
IBMX (1 mM)	1.8 \pm 0.2 (2)	1.2 \pm 0.4 (2)	0.6
Forskolin (100 μ M)	4.1 \pm 0.3 (3)	1.1 \pm 0.3 (3)	0.3
IBMX and forskolin	22.0 \pm 3.3 (3)	1.8 \pm 0.2 (3)	0.08

Explants were exposed to the various agonists and high K⁺ for 3 min at 37°C. All systems contained 1 mM IBMX, except when forskolin was added alone. The numbers shown were obtained by dividing the values obtained after stimulation by the values obtained with IBMX alone except for IBMX or forskolin, where the stimulated values are divided by the basal controls (mean fold stimulation \pm SD). The numbers in parentheses are the numbers of separate experiments each done in triplicate.

IMP was not a result of the increase in cAMP. To test whether cAMP causes any change in AMP/IMP, isoprenaline, and VIP, which stimulate adenylate cyclase via Gs, and forskolin, which largely elevates cAMP by direct activation of the catalytic unit of adenylate cyclase (Seamon and Daly, 1986), were added. All three ligands caused similar or greater increases in cAMP compared with CCh, BK, and high K⁺ but had no effect on the levels of AMP/IMP (Table 1). Furthermore, forskolin and 3-isobutyl 1-methylxanthine (IBMX) together also had no effect on AMP/IMP, although their joint activation promoted a 22-fold increase in cAMP in 3 min.

Effects of carbachol are mediated by muscarinic receptors

To determine whether the response to CCh was mediated via muscarinic (mAChR) or nicotinic (nAChR) cholinergic receptors, we examined 1) whether mAChR and nAChR antagonists block the response to CCh and 2) whether nicotine and muscarine were able to induce changes in cAMP and AMP/IMP levels. Figure 2 shows that the nAChR antagonist d-tubocurarine (50 μ M) had no effect on the responses to CCh, whereas atropine and the M1 mAChR inhibitor pirenzepine (50 μ M) effectively abolished both cAMP and AMP/IMP elevations induced by CCh (Figure 2, A and C), indicating that the mAChR are mediating these responses to CCh. Confirming these conclusions, muscarine (20 μ M) mimicked the CCh-induced rise in cAMP and AMP/IMP, whereas nicotine (20 μ M) had no stimulatory effect (Figure 2, B and D).

Rise in cAMP is dependent on extracellular calcium

High K⁺, CCh, and BK have been shown to cause a rise in [Ca²⁺]_i in neurons and related cells (Vicentini *et al.*, 1986; Jackson *et al.*, 1987; Gatti *et al.*, 1988; Thayer *et al.*, 1988b; O'Sullivan *et al.*, 1989; Villaroel *et al.*, 1989). To investigate whether a rise in [Ca²⁺]_i might be involved in mediating the increase in cAMP in response to the three stimulants, we first tested the effects of adding the Ca²⁺ chelator 1,2 bis(2-amino-phenoxy)ethane *N,N,N',N'* tetraacetic acid (BAPTA) to the test solutions to reduce [Ca²⁺]_o to <100 nM. In the presence of BAPTA, there was no increase in cAMP in response to CCh, BK, or high K⁺ (Figure 3A), suggesting that a rise in [Ca²⁺]_i was necessary to induce this response and also that this rise in [Ca²⁺]_i was dependent on Ca²⁺ influx. However, the rise in AMP/IMP in response to CCh and BK was not diminished but rather increased in the presence of BAPTA, and only the rise in AMP/IMP in response to high K⁺ was significantly inhibited. Similar results were obtained when 2 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) was used instead of BAPTA (Table 2). These results show that the rise in cAMP and AMP/IMP promoted by CCh and BK can be uncoupled. The continued depletion of ATP induced by CCh and BK in the presence of BAPTA also suggested that Ca²⁺ influx leading to a rise in [Ca²⁺]_i is not involved in this response.

CCh, BK, and high K⁺ cause a rise in [Ca²⁺]_i

We used the Ca²⁺-sensitive fluorescent dye fura-2 to measure the amplitude of the [Ca²⁺]_i rise

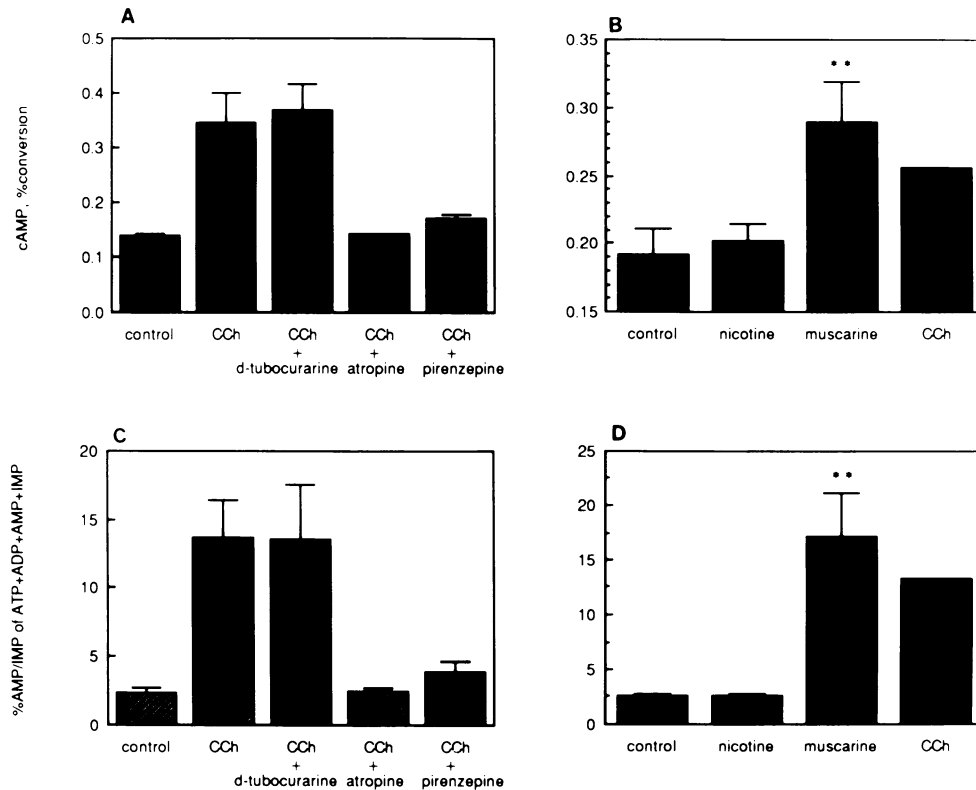


Figure 2. Muscarine but not nicotine mimics the action of CCh. The experiment was performed essentially as described in the legend to Figure 1. (A and C) CCh, 100 μ M; d-tubocurarine, atropine, and pirenzepine, 50 μ M. (B and D) Muscarine and nicotine concentrations were 20 μ M. Results are averages of triplicates \pm SD. ** $p < 0.005$.

induced by the different stimulants to determine 1) whether there was a correlation between the extent of increase in $[Ca^{2+}]_i$ and cAMP levels and 2) whether the increase in $[Ca^{2+}]_i$ was dependent on Ca^{2+} influx. In the experiment shown in Figure 4A, CCh added together with 2 mM BAPTA had no effect on $[Ca^{2+}]_i$ —although, after repeated washes, addition of CCh alone caused an increase in $[Ca^{2+}]_i$, suggesting that this rise in $[Ca^{2+}]_i$ was dependent on Ca^{2+} influx. All cells tested ($n = 10$) responded to CCh with an increase in $[Ca^{2+}]_i$. In some of the experiments, the system was calibrated so that ratio values could be converted to $[Ca^{2+}]_i$. The mean resting level of $[Ca^{2+}]_i$ was 43 ± 16 nM (29 cells, mean \pm SD), and, in the presence of 100 μ M CCh, this increased by 3.1-fold to 135 ± 26 nM (8 cells, mean \pm SD). The response to CCh consisted of two components, a nicotinic and a muscarinic component. This is shown in Figure 4B, where $[Ca^{2+}]_i$ increased in response to application of 20 μ M muscarine and then increased further on addition of 20 μ M nicotine. The additivity of the response to nicotine and muscarine varied among different cells. Figure 4D, for example,

shows that addition of muscarine after nicotine produced only a very small increase in $[Ca^{2+}]_i$. The pattern of the Ca^{2+} response to the agonist also varied (Figure 4, C and D).

Figure 5 shows typical responses elicited by 10 nM BK. Similar responses were obtained whether BK was added before or after a stimulus with high K^+ (Figure 5, A and C). When 2 mM BAPTA was added together with BK, there was no increase in $[Ca^{2+}]_i$. Altogether, 11 out of 12 cells tested responded to BK with an average fold increase in $[Ca^{2+}]_i$ of 2.5 ± 0.5 (5 cells, mean \pm SD), whereas, in all 5 experiments where BK was added in the presence of 2 mM BAPTA, there was no response. Once a cell had been exposed to BK, subsequent applications produced very little response, probably because of desensitization of the receptor. Therefore the response to BK alone and the response to BK in the presence of BAPTA had to be compared in different cells.

Compared with CCh and BK, high K^+ produced the largest increase in $[Ca^{2+}]_i$: 12.1 ± 1.7 fold (10 cells, mean \pm SD). Thus, there appears to be some correlation between the magnitude

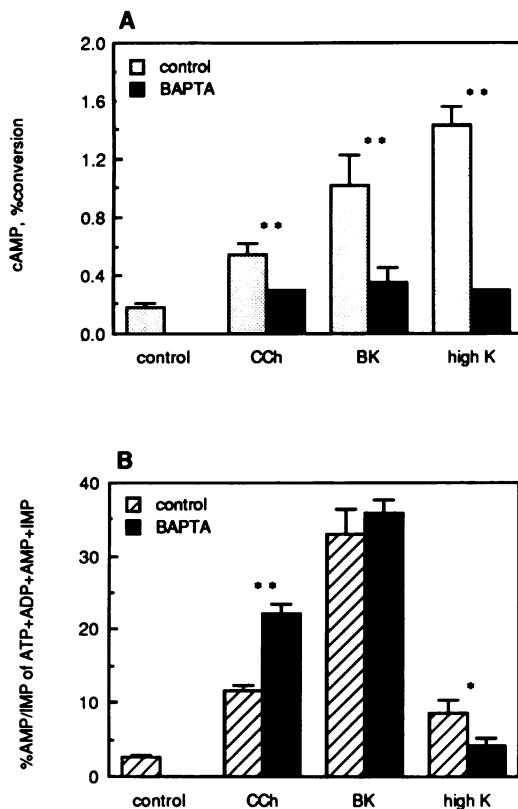


Figure 3. BAPTA blocks the agonist-mediated rise in cAMP but not the rise in AMP/IMP. The experiment was performed as described in the legend to Figure 1. BAPTA was added as indicated at 2 mM. Results are averages of triplicates \pm SD. * $p < 0.01$, ** $p < 0.005$.

of rise in $[Ca^{2+}]_i$ and the rise in cAMP levels if one takes into account that a significant fraction of the CCh-induced $[Ca^{2+}]_i$ rise is mediated via nicotinic receptors, although a causal relationship has not been established. To test whether cAMP might be causing the Ca^{2+} influx across the plasma membrane, we applied forskolin (100 μ M) or IBMX (1 mM) to cells, but neither ligand induced an increase in $[Ca^{2+}]_i$ —although all cells responded to the subsequent addition of CCh, indicating that Ca^{2+} influx is not mediated via an elevation of cAMP.

Mediators of the Ca^{2+} -dependent stimulation of adenylate cyclase

Ca^{2+} could be enhancing cAMP levels by several different mechanisms: by activating calmodulin (CaM) (Klee *et al.*, 1986), phospholipase A_2 (Van den Bosch, 1982), and/or protein kinase C (PKC) (Katada *et al.*, 1985; Yoshimasa *et al.*, 1987) or by promoting the release of noradrenaline (NA) or purines such as adenosine. A direct stimu-

lation of adenylate cyclase by Ca^{2+} -activated CaM has been observed in membranes from several cell types, notably from brain (Heideman *et al.*, 1982; Smigel, 1986). Figure 6 shows the effects of 1,3 dihydro-1-[1-[(4-methyl-4H,6H pyrrolo[1,2-a][4,1]-benzoxazepin-4-yl)methyl]-4-piperidinyl]-2H-benzimidazol-2-one (1:1) maleate (CGS-9343B), a novel selective inhibitor of CaM (Norman *et al.*, 1987). CGS-9343B (50 μ M) inhibited the rise in cAMP (Figure 6A) in response to all three stimulants, although it had no effect on basal cAMP production. It should be noted that CGS-9343B had no inhibitory effect on the rise in $[Ca^{2+}]_i$ in response to high K^+ (results not shown), indicating that it did not inhibit Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Furthermore, although elevation of $[Ca^{2+}]_i$ induced by CCh was reduced by CGS 9343B, this inhibition was $<30\%$. Thus it is possible that CaM is one component of the response pathway, although this requires further testing. Interestingly, the rise in AMP/IMP was also significantly reduced by CGS-9343B (Figure 6B), which contrasts with the inability of BAPTA (which blocked the rise in $[Ca^{2+}]_i$) to inhibit this effect.

The involvement of arachidonate metabolites in mediating the Ca^{2+} -dependent rise in cAMP was also examined with the use of indomethacin, because stimulation of the M_1 and M_4 subtypes of mAChRs and BK receptors results in potent stimulation of phosphoinositide and arachidonate metabolism in numerous systems (Bareis *et al.*, 1983; Conklin *et al.*, 1988; Peralta *et al.*, 1988; Perney and Miller, 1989) and possibly in SCG (Bone and Michell, 1985; Horwitz *et al.*, 1985). Indomethacin (50 μ M) partially inhibited the rise in cAMP evoked by CCh and BK but had little effect on the high K^+ -induced rise in cAMP (Figure 6, B and D). The block of the BK- and CCh-induced rise in cAMP by indomethacin was quite variable, ranging from 40 to 80% in different experiments. The BK- and CCh-evoked rise in AMP/IMP was also reduced by indomethacin, but no effect was observed in the presence of high K^+ . Thus, the effects of BK and CCh on cAMP and ATP metabolism may be mediated in part by cyclo-oxygenase-derived metabolites, whereas high K^+ does not appear to activate this pathway. We also examined the effects of the PKC agonist phorbol 12-myristate 13-acetate (10–100 nM), but no change in cAMP or AMP/IMP was observed, although the same solution enhanced the release of [^{14}C]noradrenaline evoked by high K^+ by $52 \pm 8\%$ (2 experiments in triplicate, mean \pm SD). The same solution also stimulated phosphati-

Table 2. Inhibition or stimulation of CCh, BK, and high K⁺-induced responses by EGTA or BAPTA, CGS-9343B or indomethacin

	CCh	BK	High K ⁺
	$100 \times \frac{(\text{Stimulant} + \text{Inhibitor} - \text{Background})}{(\text{Stimulant} - \text{Background})}$		
cAMP			
EGTA or BAPTA	13 ± 3	14 ± 7	6 ± 2
CGS-9343B	0 ± 8	11 ± 4	19 ± 6
Indomethacin	52 ± 11	50 ± 2	100 ± 31
AMP/IMP			
EGTA or BAPTA	245 ± 68	120 ± 30	26 ± 11
CGS-9343B	85 ± 23	12 ± 5	21 ± 8
Indomethacin	82 ± 31	43 ± 23	124 ± 33

The results in Figures 2, 6, and 7 were combined with those of other experiments (1–2 separate experiments for CGS-9343B and indomethacin, 3–4 separate experiments for EGTA and BAPTA) to yield average net inhibition or stimulation by the three inhibitors. The numbers were calculated by dividing the values obtained in the presence of the stimulant and inhibitor by the values obtained in the absence of inhibitor after subtracting the average background values 0.2 ± 0.05% conversion for cAMP, 2.6 ± 0.5% AMP/IMP) obtained in the absence of the stimulant. None of the inhibitors had an effect on the basal levels of cAMP or AMP/IMP. Numbers are 100 × mean net ratios ± SD.

dylcholine synthesis in fibroblasts in five separate experiments by threefold (Suidan and Tolkovsky, in preparation). A comparison of the net inhibition or stimulation of cAMP synthesis and ATP degradation caused by EGTA or BAPTA, CGS-9343B, and indomethacin is summarized in Table 2.

Is elevation of cAMP due to NA release?

Several experiments were conducted to test whether the rise in cAMP was due to an autoid action of substances released from the neurons. Figure 7 shows that the addition of CCh in the absence or presence of atropine, which was included to block a possible muscarinic inhibitory mechanism (Wanke *et al.*, 1987), caused no measurable neurotransmitter release, whereas high K⁺ and BK each caused a 2- to 3-fold increase in NA release over basal levels, and, when added together, their responses were additive. Thus, it is unlikely that CCh causes either cAMP or AMP/IMP elevation by releasing neurotransmitter. However, the results do not rule out the possibility that the rise in cAMP in response to BK or high K⁺ was secondary to a constitutive release of NA, which could interact with β -adrenergic receptors coupled to Gs, for example. However, the antagonist propanolol (10 μ M) had no inhibitory effect, although it did block the isoprenaline-induced rise in cAMP. Although we did not test the ef-

fects of α -adrenergic antagonists, the addition of the α -adrenergic receptor agonist phenylephrine (10 μ M) had no effect on either cAMP or ATP levels. ATP, which is released from vesicles along with NA, also had no effect on the levels of cAMP when added alone. Because increased ATP breakdown leads to the release of adenosine from these cells (Tolkovsky and Suidan, 1987), we also examined whether adenosine induces a rise in cAMP. Although adenosine (50 μ M) caused a small rise in cAMP (from 0.2 to 0.25% conversion, mean ± SD, n = 3), this increase was much lower than the 2.2- to 5.8-fold increase in cAMP observed in response to CCh, BK, or high K⁺. Moreover, the concentration of released adenosine would be well under 50 μ M. Thus it is unlikely that the elevation of cAMP is due to the activation of β -adrenergic receptors by released NA, ATP, or adenosine, although the possibility that released NA could activate α -adrenergic receptors was not excluded.

Cellular localization of cAMP and ATP responses

The ability of nicotine to cause a rise in [Ca²⁺]_i in the cell bodies without affecting the levels of cAMP or AMP/IMP suggested that elevation of [Ca²⁺]_i was not sufficient to cause a response. It is also possible, however, that the responses to the stimulants may require elevation of [Ca²⁺]_i.

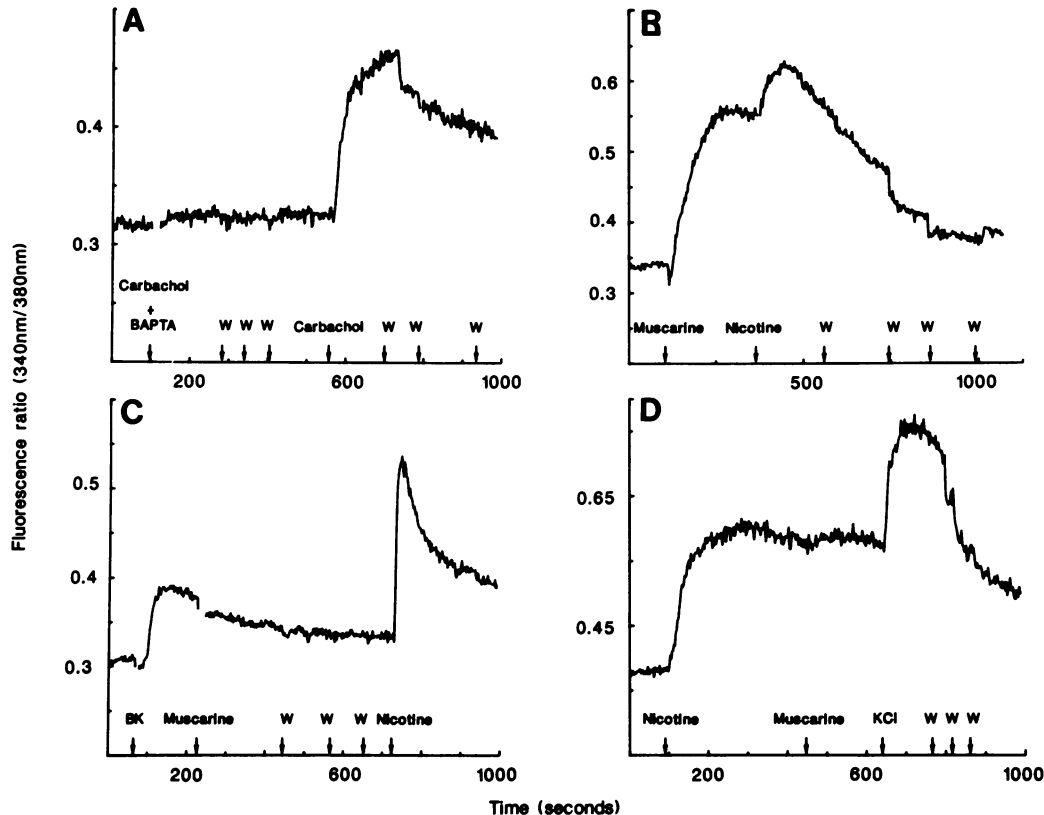


Figure 4. $[Ca^{2+}]_i$ is elevated by CCh, muscarine, and nicotine and is blocked by BAPTA. Neurons cultured for 13 d were loaded with fura-2 in a thermoregulated chamber for 15 min and then washed in L15 medium buffered with 10 mM HEPES-NaOH, pH 7.4, at 37°C. W signifies a wash with L15 medium. 340/380 ratios were calculated every 50 ms. (A) CCh (100 μ M) together with BAPTA (2 mM) was added to chamber for 3 min. After three washes with L15 medium, CCh (100 μ M) was added alone for 2–3 min and then washed out. (B) Muscarine (20 μ M) was added for 3 min followed without washing by nicotine (20 μ M) for 2 min. (C) BK (10 nM) was added for 2 min followed without washing by muscarine (20 μ M). After three washes, nicotine (20 μ M) was added. (D) Nicotine (20 μ M) added for 6 min followed without washing by muscarine (20 μ M) and 3 min later by KCl (50 mM).

in different cellular compartments (O'Sullivan *et al.*, 1989). Neurites of 2- to 3-wk explants contain >80% of cellular adenylate cyclase activity (Tolkovsky, 1987) and ~65% of total cellular ATP (Tolkovsky and Suidan, 1987). We therefore were interested to see whether the site of cAMP production and ATP decrease colocalize to the same compartment in the neurons and to examine whether stimulation of cell bodies was important for cAMP production. The cell bodies were therefore removed before or after stimulation with CCh, BK, or high K^+ . As shown in Figure 8, BK and high K^+ were equally effective in eliciting responses when neurites were detached from the cell body before stimulation. In the case of CCh, however, there was less than the expected increase in cAMP and AMP/IMP when neurites were detached from the cell bodies before stimulation, and higher cAMP levels were found in neurites attached to the

cell bodies during stimulation, suggesting that stimulation at the cell body may enhance the effects of CCh at the neurites.

Discussion

We have shown that CCh (by activating mAChR) and BK induce significant activation of adenylate cyclase in rat sympathetic neurons (Figures 1 and 2). The rise in cAMP is clearly dependent on the presence of extracellular Ca^{2+} and is correlated with the magnitude of the rise in $[Ca^{2+}]_i$ (Figures 3–5, Table 2), suggesting that Ca^{2+} influx is required. Activation of CaM may be involved, because the increase in cAMP was blocked by CGS-9343B (Figure 6, Table 2) at concentrations that permit the CCh- and high K^+ -induced rise in $[Ca^{2+}]_i$ in the cell bodies (Suidan, 1989) and PKC activity (Norman *et al.*, 1987; Hill *et al.*, 1988). Products of cyclo-oxygenase

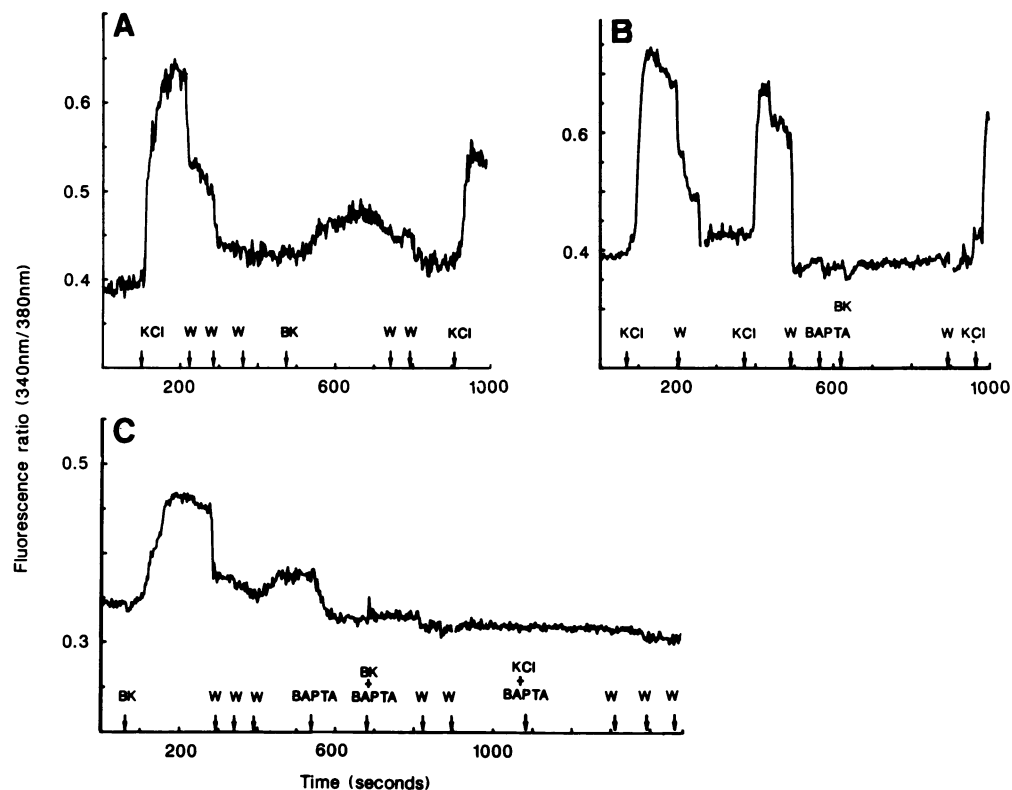


Figure 5. $[Ca^{2+}]_i$ is elevated by BK and is blocked by BAPTA. Neurons grown for 13 d were loaded with fura-2 and washed as described in the legend to Figure 4. (A) KCl (50 mM) added for 2 min followed by three washes. BK (10 nM) was then added for 4 min then washed out, and KCl (50 mM) was added again. (B) Two additions of KCl (50 mM) separated by one wash, followed by addition of BK (10 nM) together with BAPTA (2 mM) for 5 min. After one wash with L15 medium, KCl (50 mM) was added again. (C) BK (10 nM) added for 3 min and washed out; then BAPTA (2 mM) was added, followed by BK (10 nM) and BAPTA (2 mM). After two washes, KCl (50 mM) was added together with BAPTA (2 mM).

activities also mediate part of the agonist-induced rise in cAMP, because this can be partially (or totally in some experiments with BK) blocked by the cyclooxygenase inhibitor indomethacin (Figure 6, Table 2).

In addition to the rise in cAMP, we have shown that BK and CCh cause an apparent metabolic stress, where the rate of ATP utilization is not matched by its rate of synthesis. This effect is much larger than that evoked by high K^+ . We have localized the response site to the neurites where BK, but not CCh, also promotes the release of NA in a manner that is additive with that of high K^+ .

A unifying hypothesis for high K^+ , BK, and CCh actions

It is unlikely that BK and mACh receptors are coupled to adenylate cyclase via G_s , because their effects, like those of high K^+ , require elevation of $[Ca^{2+}]_i$, whereas stimulation of adenylate cyclase by isoprenaline and VIP does not

depend on Ca^{2+} , nor do these agonists deplete ATP (Table 1). There are, however, differences among the actions of CCh, BK, and high K^+ , and, to account for these differences, we propose the following working hypothesis: all three stimulants activate Ca^{2+} influx, CaM, and arachidonate metabolism but to varying degrees, the more dominant element determining the extent of rise in cAMP and AMP/IMP, the relative sensitivities to the blockers indomethacin and CGS-9343B, and the ability to evoke neurotransmitter release. In the case of high K^+ , the influx of Ca^{2+} is sufficiently large to be dominant; thus, reduction of $[Ca^{2+}]_i$ with BAPTA blocks the substantial rise in cAMP evoked by high K^+ , the small rise in AMP/IMP, and the release of NA. In agreement, CGS-9343B has similar effects to BAPTA, whereas indomethacin has very little effect on either the rise in cAMP or AMP/IMP.

For BK, the contribution of the arachidonate metabolites is dominant, especially with regard to ATP metabolism; thus, the rise in both cAMP

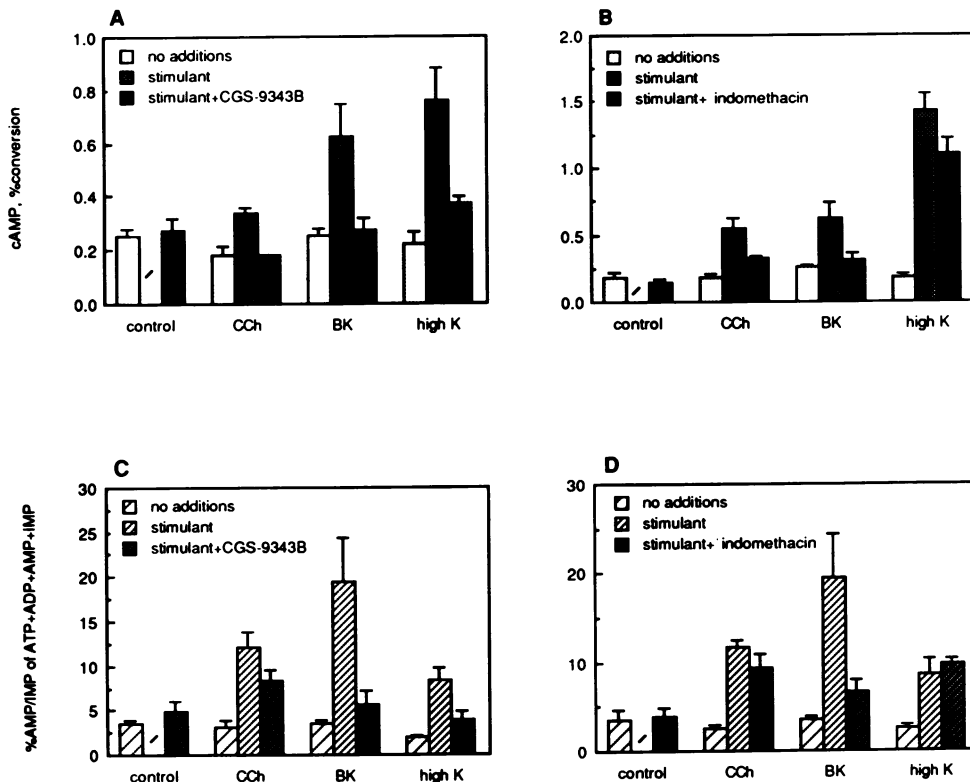


Figure 6. Inhibition of the responses to CCh, BK, and high K^+ by CGS-9343B and indomethacin. Explants loaded with [3H]adenine were stimulated for 3 min at $37^\circ C$ with $100 \mu M$ CCh, $10 nM$ BK, or $54 mM$ KCl in the presence of $50 \mu M$ CGS-9343B (A and C) or $50 \mu M$ indomethacin (B and D). When indomethacin was used, explants were also washed for 15 min in its presence before stimulation. All systems contained $1 mM$ IBMX and 0.4% DMSO. Results are averages of triplicates \pm SD.

and AMP/IMP is inhibited by indomethacin, whereas blocking the increase in $[Ca^{2+}]_i$ with BAPTA causes no inhibition of AMP/IMP increase. However, activation of CaM must also be necessary for cAMP elevation, because blocking either Ca^{2+} influx or inhibiting CaM with CGS-9343B also abolishes this rise. Although BK evokes only a small rise in $[Ca^{2+}]_i$, this could be sufficient to act synergistically with the arachidonate metabolites to promote NA release (Rindlisbacher *et al.*, 1990). It is clear that the relevant BK receptors are mostly localized at the neurites (Figure 8), as cAMP was elevated to the same extent in the neurites whether they were separated from cell bodies before or after stimulation.

To explain the actions of CCh, we propose that the combined nicotinic and muscarinic actions of CCh are less effective than high K^+ in elevating $[Ca^{2+}]_i$ in the neurites and that activation of muscarinic receptors in the neurites is also less effective than BK in eliciting arachidonate metabolism. Hence, CCh induces a smaller rise in both cAMP and AMP/IMP than

either high K^+ or BK, and the small rise in neuritic $[Ca^{2+}]_i$ fails to promote detectable NA release because of the lack of arachidonate metabolites. However, CCh may also induce another signal not shared by BK or high K^+ , because the CCh-induced rise in AMP/IMP is actually potentiated by BAPTA (or EGTA) and is inhibited only 20% by CGS-9343B. The muscarinic actions of CCh are most likely mediated via M_1 or M_4 mAChR subtypes (Bonner, 1989; Felder *et al.*, 1989; Ramachandran, 1989) because these have been shown to stimulate cAMP production (as well as phosphoinositide metabolism) with an EC_{50} ($8 \pm 2 \mu M$) comparable with that found in cell lines transfected with M_1 or M_4 mAChRs (Peralta *et al.*, 1988; Conklin *et al.*, 1988).

Mechanism of ATP depletion

The observation that BK and mAChR activation enhance ATP depletion is novel. It is clear that the decrease in ATP is not due to cAMP elevation because isoprenaline, VIP, and forskolin, even when added with IBMX, had no effect on

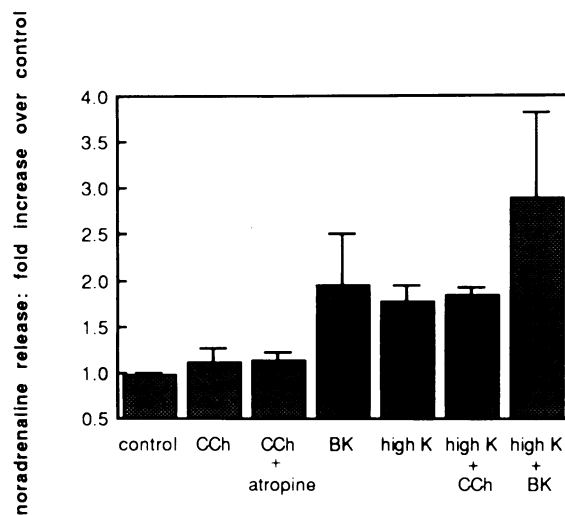


Figure 7. Release of [14 C]NA in response to CCh, BK, and high K^+ . Explants preincubated with $41.7 \mu\text{M}$ [14 C]NA for 2 h were washed and subjected to a series of applications of 3 min duration as described in Materials and methods. The results (mean \pm SD of triplicate determinations) are shown as the ratio of the release in the presence of the stimulus to the release measured during the preceding control application. Basal release over 3 min was $2 \pm 1\%$ of total uptake. CCh, $100 \mu\text{M}$; atropine, $1 \mu\text{M}$; BK, 14 nM ; and KCl, 54 mM .

ATP levels. Nor is it caused by the rise in $[\text{Ca}^{2+}]_i$, per se, because the decrease in ATP induced by CCh and BK was larger than that induced by high K^+ and it persisted in the presence of BAPTA. We have proposed, therefore, that a major part of ATP degradation is caused by the liberated arachidonate metabolites, perhaps by causing uncoupling of the electron-transfer mechanism in mitochondria similar to the actions of fatty acids. However, another possible cause for the increased ATP breakdown, which we are presently exploring, is a rise in $[\text{Na}^+]_i$, resulting in activation of the Na^+-K^+ ATPase, which is known to be the major sink for ATP in neural tissues (Pull and McIlwain, 1972). Indeed, in the presence of veratridine ($20 \mu\text{M}$), the relative amount of [^3H]ATP was reduced from 74 ± 3.5 to $30.7 \pm 2.2\%$ and the relative amount of [^3H]AMP/IMP increased from 6.1 ± 1.3 to $24.3 \pm 1.7\%$ within 10 min (Suidan, 1989). Both BK and CCh may activate nonselective Na^+ -permeant cation channels of the types described by Matsumoto and Pappano (1989) for CCh and by Fasolato *et al.* (1988) for BK. The observation that such channels remain active over several minutes, whereas voltage-gated Na^+ channels activated by high K^+ inactivate within a few milliseconds (R. Murrell, unpublished), would account for the lesser efficacy of

high K^+ in elevating AMP/IMP. Furthermore, some ligand-activated Ca^{2+} channels may become Na^+ permeant in the presence of Ca^{2+} chelators (Kostyuk, 1980), which would explain why the AMP/IMP levels are enhanced in the presence of BAPTA or EGTA, which completely blocked elevation of $[\text{Ca}^{2+}]_i$. Although it is well established that CCh and BK activate phospho-

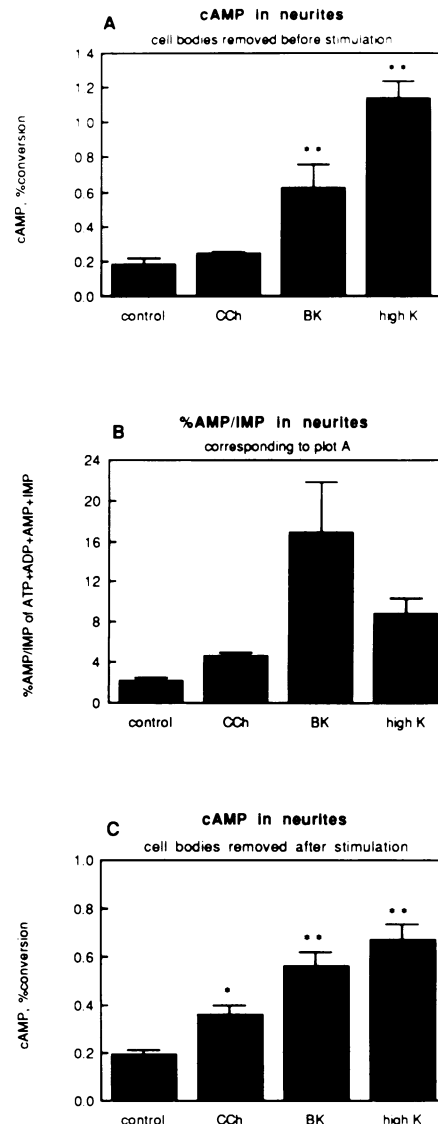


Figure 8. Localization of stimulated cAMP production in neurites and cell bodies. The experiment was performed essentially as described in the legend to Figure 1, but the cell bodies were detached from the explants either before (A and B) or after (C) stimulation with the agonist as previously described (Tolkovsky, 1987). To prevent leakage of metabolites from explants dissected after stimulation, explants were rapidly frozen on dry ice before removal of the cell bodies and cut frozen.

inositide metabolism and the release of Ca^{2+} from intracellular stores in several types of neurons and related cells (Bone *et al.*, 1984; Bone and Michell, 1985; Horwitz, 1985; Fasolato *et al.*, 1988; O'Sullivan *et al.*, 1989), the observation that BAPTA (or EGTA) completely blocked the CCh- and BK-induced rises in $[\text{Ca}^{2+}]_i$ suggests that the contribution of the Ca^{2+} stores to the rise in $[\text{Ca}^{2+}]_i$ in these neurons is relatively small, as suggested by Thayer *et al.* (1988a). If Ca^{2+} is released from intracellular stores, a constant supply of Ca^{2+} from the medium is clearly required to sustain the rise in $[\text{Ca}^{2+}]_i$ over a 3-min period (Figures 4 and 5). The influx of Ca^{2+} may be mediated by inositol (1,3,4,5) tetrakisphosphate (Irvine and Moor, 1986; Nahorski, 1988). Alternatively, muscarinic and/or BK receptors may be coupled directly to a Ca^{2+} -permeant cation channel of the type mentioned by Brown *et al.* (1989). These possibilities remain to be explored.

Conclusions

We have described two novel effects of the agonists CCh and BK, the actions of which are commonly attributed to phospholipase C activation: an elevation of cAMP and a reduction of ATP by as much as 40%. The interpretation of how these agonists promote both short- and long-term changes in key cellular activities may have to be modified in light of these results.

Materials and methods

CGS-9343B was a gift from CIBA-GEIGY (Summit, NJ); CCh, muscarine, BK acetate, isoprenaline, atropine sulphate, indomethacin, pirenzepine, and IBMX were obtained from Sigma (St. Louis, MO); nicotine hydrogen tartrate was from BDH (Poole, UK); forskolin was from Calbiochem (San Diego, CA); and BAPTA was a gift from Dr. G. Smith (Cambridge University, Cambridge, UK) or from Calbiochem.

Cell culture

Explants or isolated neurons prepared from SCG of 1- to 2-d-old Wistar rats were cultured in enriched L15 (GIBCO, Paisley, UK) (Hawrot and Patterson, 1979) containing 5% rat serum and 3–4 nM 2.5 S NGF (Tolkovsky and Suidan, 1987). Explants of ~200 μm diam and isolated neurons were plated on 13-mm glass coverslips precoated with collagen (~0.3 O.D. at 280 nm) and grown in an incubator in a water-saturated 5% CO_2 -air environment at 37°C. All explants were used at between 2 and 3 wk in culture. In experiments performed in the absence of neuronal cell bodies, the explant was excised from the culture as described previously (Tolkovsky, 1987).

Determination of cAMP and other adenine-based metabolites

Changes in cAMP production were measured after metabolic labeling of the adenine-based metabolites with $[2\text{-}^3\text{H}]\text{adenine}$. All the experiments were carried out in the

presence of 1 mM IBMX, which inhibits >95% of the phosphodiesterase activity (Tolkovsky and Suidan, unpublished observations). IBMX was added with 0.4% dimethyl sulfoxide (DMSO), which had no detectable effect on cAMP or ATP metabolism. Explants grown for 2–3 wk were incubated 12–15 h in growth medium containing 1.9 μM $[^3\text{H}]\text{adenine}$ (26 Ci/mmol). The specific activity of ATP attained was 6.2 Ci/mmol (Tolkovsky and Suidan, 1987), which enabled the detection of ~2–4 fmol of cAMP above a background of 27 ± 10 dpm. This was more than sufficient because the amount of labeled cAMP normally found in an extract of nonstimulated explant was 7.3 ± 1.1 fmol/explant ($n = 29$). Comparison of the $[^3\text{H}]\text{cAMP}/[^3\text{H}]\text{ATP}$ ratio determined by this method to that of total cAMP measured by radioimmunoassay and total ATP measured luminometrically was $8.2 \pm 2.1 \times 10^{-4}$ ($n = 29$) and $8.7 \pm 2.9 \times 10^{-4}$ ($n = 11$), respectively. Moreover, the increase in cAMP in response to 100 μM forskolin (2.4 ± 0.3 -fold vs. 3.1 ± 0.32 -fold), 1 mM IBMX (1.8 ± 0.2 -fold vs. 1.7 ± 0.28 -fold) or both (50.1 ± 2.6 -fold vs. 26.6 ± 16.7 -fold) was also similar between the radiometric and radioimmunoassay methods, indicating that the labeling procedure reports changes in cAMP with high fidelity. After incubation, cultures were washed three times for 5 min at 37°C in 3 ml of an L15-based plating medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–NaOH (HEPES–NaOH), 5 mM KCl, and 1.2 mM Ca^{2+} , pH 7.4. All experiments were performed in this solution, except when depolarized with high K^+ , in which case the plating medium was diluted with a solution containing 150 mM KCl, 1.2 mM CaCl_2 , and 0.6% glucose to produce 54 mM KCl without altering Ca^{2+} or glucose concentrations. Three coverslips containing explants were placed in one well containing 3 ml test solution at 37°C for 3 min, after which coverslips were blotted and dropped individually into test tubes containing 300 μl 15% aqueous acetic acid and 600 μl chloroform on ice. Tubes were mixed vigorously and spun; the contents were transferred to an Eppendorf tube, spun again, and put back on ice. To separate the adenine-based metabolites, 20- μl aliquots of the aqueous phase were spotted gradually under a stream of cold air onto polyethyleneimine-cellulose TLC plates (UV254, Machery-Nagel) that were divided by scoring with a sharp object into 1.5×20 -cm lanes, and a similar total volume was spotted separately on 2.5×1 -cm strips to determine recovery. Before spotting the samples, we spotted 2.5 μl of markers (9 mM each of ATP, ADP, AMP, IMP, cAMP, NAD, adenosine, hypoxanthine, and inosine) onto the origin of each lane. The metabolites were separated by a two-step elution: 1) 5 cm in *n*-butanol:methanol:water 1:1:8 followed without drying by 15 cm in water. Plates were dried and an ~2-cm strip from the top containing impurities from the plate (but no radioactivity) was removed; 2) 12 cm in 0.3 M LiCl and ~6 cm in 1 M LiCl. The $R_f \times 100$ values of the separated metabolites were ATP 2, ADP 7, AMP/IMP 24, cAMP 51, NAD 68, adenosine/hypoxanthine 78, and inosine 89. Strips containing nucleotides were cut and eluted in scintillation vials containing 0.5 ml of a 100:2 mixture of 0.7 M MgCl_2 :1M tris(hydroxymethyl)aminomethane (Tris)–HCl pH 7.4, followed by 4 ml scintillant (Optiphase Hi safe, LKB, Uppsala, Sweden). Vials were counted after 24 h to allow complete elution of the nucleotides.

Measurement of $[\text{Ca}^{2+}]_i$

Neurons were loaded with fura-2 at 37°C for 15 min in L15-HEPES medium (pH 7.4) containing 10 mM HEPES, 0.6% glucose, and 2 μM Fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR) diluted from a stock solution (1 mM) containing 20% pluronic F-127 in DMSO on a heated stage

of an epifluorescence microscope (Nikon Diaphot, Garden City, NY). Cells were washed three times in 1 ml of L15-HEPES for 2 min each. $[Ca^{2+}]_i$ was measured in cell bodies at 37°C using a Deltascan system with dual excitation and single emission (Tolkovsky *et al.*, 1990). Excitation at 340 or 380 nm changed every 25 ms, and emission was collected at 511 nm with a photomultiplier tube. The plots show 20 points/s. By use of the equation of Grynkiewicz *et al.* (1985), $[Ca^{2+}]_i$ was approximated by measuring fluorescence of a solution containing 0.25 and 0.5 μ M fura-2 free acid and either 1 mM EGTA (pH 7.4) or 10 mM free $[Ca^{2+}]$ (pH 7.4). Calibration of fluorescence ratios by titration of 50 μ M fura-2 in the presence of 1 mM BAPTA and known $[Ca^{2+}]$ (similar to Thayer *et al.*, 1988a,b) established that resting $[Ca^{2+}]_i$ in the absence of serum was 43 ± 16 nM ($n = 29$, SD). Drugs were added at two- to threefold the final concentration to obtain rapid mixing.

Noradrenaline release

Explants (2–3 wk in culture) grown in 17-mm wells were incubated in growth medium containing 41.7 μ M [^{14}C]NA (60 mCi/mmol) and 10 μ g/ml ascorbate for 2 h, washed, and subjected to a series of 3-min applications of control and test solutions (300 μ l) essentially as previously described (Tolkovsky and Suidan, 1987). To measure NA release, a sample of each solution was removed and counted.

Statistical analysis

The Student's *t* test was used to test the significance of the differences between the means. In the figures, all the results are mean \pm SD, **p* < 0.01, ***p* < 0.005. Where no error bar is shown, it is too small to be resolved.

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